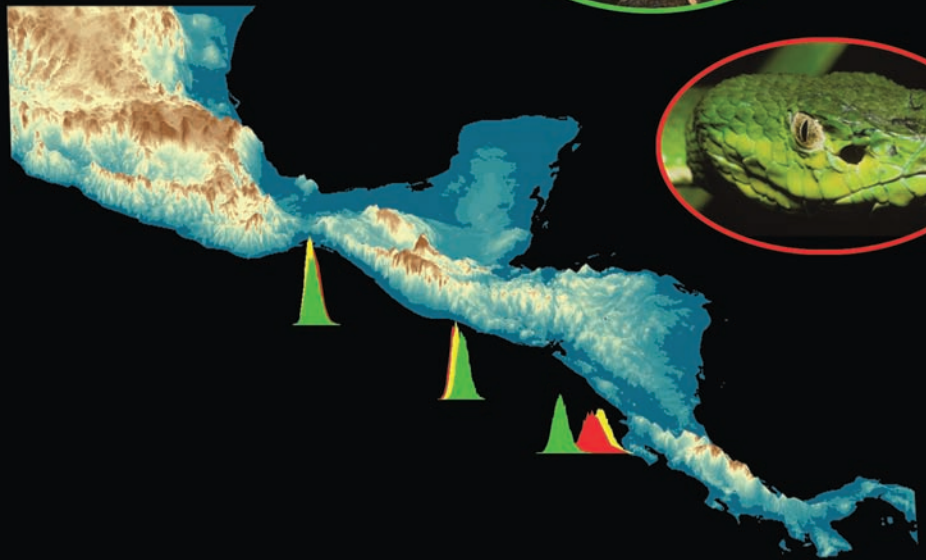


ISSN 0305-0270

Volume 36
Number 1
January 2009

Journal of Biogeography

Editor-in-Chief: Robert J. Whittaker



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BLACKWELL



Comparative phylogeography of pitvipers suggests a consensus of ancient Middle American highland biogeography

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ABSTRACT

Aim We used inferences of phylogenetic relationships and divergence times for three lineages of highland pitvipers to identify broad-scale historical events that have shaped the evolutionary history of Middle American highland taxa, and to test previous hypotheses of Neotropical speciation.

Location Middle America (Central America and Mexico).

Methods We used 2306 base pairs of mitochondrial gene sequences from 178 individuals to estimate the phylogeny and divergence times of New World pitviper lineages, focusing on three genera (*Atropoides*, *Bothriechis* and *Cerrophidion*) that are broadly co-distributed across Middle American highlands.

Results We found strong correspondence across three highland lineages for temporally and geographically coincident divergences in the Miocene and Pliocene, and further identified widespread within-species divergences across multiple lineages that occurred in the early–middle Pleistocene.

Main conclusions Available data suggest that there were at least three major historical events in Middle America that had broad impacts on species divergence and lineage diversification among highland taxa. In addition, we find widespread within-species genetic structure that may be attributable to the climatic changes that affected gene flow among highland taxa during the middle–late Pleistocene.

Keywords

Atropoides, *Bothriechis*, *Cerrophidion*, Mexico, Miocene, montane forests, Neotropics, Pleistocene, speciation, Viperidae.

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INTRODUCTION

Phylogenetic inferences coupled with robust estimates of divergence times can provide tremendous insight into the patterns and underlying causes of the historical diversification of lineages. Despite the power of such inferences, however, it is difficult to determine to what extent any single biogeographical example may be broadly representative of the patterns exhibited by diverse biotic components of a region or ecosystem. By comparing and contrasting phylogeographical scenarios from co-distributed lineages, comparative phylogeography (Bermingham & Martin, 1998; Bermingham & Moritz, 1998; Avise, 2000; Sullivan *et al.*, 2000; Lapointe & Rissler, 2005; Hickerson *et al.*, 2006) provides further understanding by identifying biogeographical patterns and the extent

to which these apply to various taxa. If multiple lineages appear to be subject to spatially and temporally congruent patterns of divergence, a more powerful inference of the major events that have had a broad impact on multiple lineages of co-distributed species can be made (Rosen, 1978; Nelson & Platnick, 1981). Deductions from comparative phylogeographical analyses are particularly important and enlightening for areas with either vague geological or tectonic information, or where little historical consensus is available (Arbogast & Kenagy, 2001; Riddle & Hafner, 2006).

Middle America, the zone extending from central Mexico through Panama (Fig. 1), is extremely biodiverse, and a large component of this diversity is endemic (Savage, 1982; Campbell, 1999). Although this region spans *c.* 16° latitude, the landmass is fairly small (*c.* 2.5 million km²), rendering its high

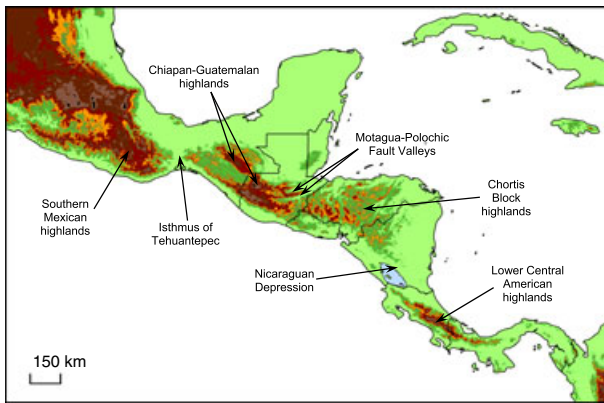


Figure 1 Map of Middle America showing the main highland regions and putative biogeographical barriers for highland taxa (based on NASA Shuttle Radar Topography Mission).

endemism most impressive (Campbell, 1999). The exaggerated topography, the interdigitation of diverse habitats, and the dynamic tectonic and climatic history of the region have contributed synergistically to its high endemism and diversity (Whitmore & Prance, 1987; Jackson *et al.*, 1996; Campbell, 1999). Middle America has experienced a complex tectonic and geological history, and lies at the active junction of four major tectonic plates and several tectonic blocks (Iturralde-Vinent, 2006; Marshall, 2007). Deciphering the events that have historically shaped present-day biological diversity is complicated due to the continual physiographical reshaping of the region since the Cretaceous. Despite substantial progress over the past several decades, the details of much of the tectonic history of Middle America remain fragmentary and controversial (Coney, 1982; Iturralde-Vinent, 2006; Mann *et al.*, 2007).

A majority of biogeographical studies concerning Middle America have been focused on understanding this region's role in biotic dispersal between North and South America, in many cases neglecting endemic patterns of Middle American biodiversity. Accordingly, most studies have dealt with biogeographical patterns in the late Pliocene–Pleistocene relating to the establishment of the final land connection with South America (Stehli & Webb, 1985; Hafner, 1991; Webb, 1997), and relatively few have investigated earlier patterns in the Miocene and early Pliocene using contemporary phylogenetic data and analyses (Bermingham & Martin, 1998; Parra-Olea *et al.*, 2004; Pennington & Dick, 2004; Crawford & Smith, 2005; Ribas *et al.*, 2005; Barker, 2007; Crawford *et al.*, 2007; Heinicke *et al.*, 2007).

Several early broad-scale studies on the biogeographical history of Middle American fauna have shaped current perceptions of the historical patterns and processes that had an impact on the regional fauna (Dunn, 1931; Duellman, 1966; Savage, 1966, 1982; Stuart, 1966). In particular, Savage (2002) proposed a model for highland speciation in Middle America in which highland species diversity was primarily the result of climatic cycles beginning in the late Pliocene and extending

through the Pleistocene. Savage proposed that subsequent to the dispersal of Nearctic lineages to Middle America in the Miocene–Pliocene, speciation in the highlands occurred as a combination of mountain uplift and fluctuations in climate during Pleistocene glacial periods (see also Savage, 2002: 830). However, these studies lacked: (i) recent geological and tectonic insights into the region's history, (ii) robust and detailed phylogenetic estimates, and (iii) explicit estimates of divergence times independent of the assumptions of a strict molecular clock.

Pitvipers represent an ideal model system for investigating historical patterns of Neotropical diversification. This large group of venomous snakes has a relatively well known phylogeny (Parkinson *et al.*, 2002; Malhotra & Thorpe, 2004; Castoe & Parkinson, 2006) and an extensive fossil record in the USA (reviewed by Holman, 2000), and appears to have dispersed into the New World as a single lineage from Asia during the Miocene (Kraus *et al.*, 1996; Parkinson, 1999; Parkinson *et al.*, 2002; see also Holman, 2000). Pitvipers are also good models for comparative phylogeography because several distinct and diverse lineages are broadly co-distributed, and extrinsic temporal constraints for divergence time estimates are available. Furthermore, because relaxed clock inferences of the relative divergence times within a single tree are particularly robust to the assumptions of calibration points (Thorne & Kishino, 2005), pitvipers are ideal for testing hypotheses of coincident divergence among multiple lineages.

Several studies have examined biogeographical hypotheses for Neotropical pitviper lineages (Crother *et al.*, 1992; Zamudio & Greene, 1997; Parkinson *et al.*, 2000; Wüster *et al.*, 2002; Gutberlet & Harvey, 2004; Werman, 2005), but have resulted in little explicit consensus. Most of these studies provided brief comments on biogeography (Kraus *et al.*, 1996; Parkinson, 1999; Parkinson *et al.*, 2002) or employed limited phylogenetic or phylogeographical data with no explicit temporal component (Crother *et al.*, 1992; Castoe *et al.*, 2003; Werman, 2005), or with temporal estimates derived from a strict molecular clock (Zamudio & Greene, 1997; Wüster *et al.*, 2002). In this study, we compare historical biogeographical patterns simultaneously across three lineages of Neotropical pitvipers that are broadly co-distributed across the highlands of Middle America. These include members of the genera *Cerrophidion* (the montane pitvipers), *Atropoides* (the jumping pitvipers) and *Bothriechis* (the palm pitvipers).

To test the highland speciation model proposed by Savage (2002) and previous hypotheses of Middle American biogeography/phylogeography, we used a large molecular phylogenetic data set for pitvipers that includes a dense (including intraspecific) sampling of members of the three genera of interest. We added new DNA sequences from members of the genera *Atropoides* and *Cerrophidion* to the data available for Neotropical pitvipers. We also estimated lineage divergence times based on multiple flexible approaches to provide a robust and probabilistic temporal component, avoiding assumptions of a strict molecular clock. We synthesize these inferences to address four questions: (1) Is the Savage

speciation model supported by highland pitviper phylogeography? This model predicts that Middle American highland species diverged from one another primarily during the late Pliocene and Pleistocene, when dramatic fluctuations in temperature may have affected highland habitat connectivity. (2) Is there evidence that temporal and geographical patterns of divergence are shared among multiple co-distributed highland lineages, and is there evidence of underlying geological or climatic causes? (3) Is there phylogeographical signal apparent from highland pitvipers that can be used to formulate an explicit model of Middle American highland speciation? (4) What effects did glacial cycles (in the late Pliocene–Pleistocene) have on lineage diversification in highland pitvipers of Middle America?

METHODS

Taxon sampling and laboratory methods

Because our goals included inferences of biogeographical patterns ranging from ancient (Miocene) to recent in multiple pitviper lineages, we incorporated a large mitochondrial DNA sequence data set (including 178 terminals) designed to provide accurate phylogenetic and divergence time estimates across this range of time. We combined mitochondrial DNA sequences from several studies (Parkinson, 1999; Malhotra & Thorpe, 2000, 2004; Parkinson *et al.*, 2002; Castoe *et al.*, 2003, 2005; Castoe & Parkinson, 2006) to include representatives of Old World pitvipers, and extensive sampling of all major New World lineages. The data set included sequences of four mitochondrial gene fragments: portions of the 12S and 16S rRNA genes and the protein coding genes NADH dehydrogenase subunit four (ND4) and cytochrome *b* (*cyt b*), for a total of 2306 aligned nucleotide positions. This included sequences for all four genes for a vast majority of species, and essentially all major lineages, although some intraspecific samples included only sequences of the two protein-coding genes ND4 and *cyt b* (1386 bp; for details see Appendix S1 in Supplementary Material).

We included all inter- and intraspecific sampling available from previous studies for the three genera of interest: *Atropoides*, *Bothriechis* and *Cerrophidion*. All taxonomic references in this study follow Campbell & Lamar (2004). We also added new sequences for 19 samples of *Atropoides* and *Cerrophidion* (Appendix S1). Laboratory methods for generating new sequences followed Parkinson *et al.* (2002), Castoe *et al.* (2005) and Castoe & Parkinson (2006), as did sequence-alignment methods.

Phylogenetic analysis

Aside from the relatively small number of new intraspecific sampling added in this study, the data used here essentially represent the combination of data sets from Castoe *et al.* (2005) and Castoe & Parkinson (2006), with the exclusion of some fine-scale sampling of Old World pitvipers. To infer

phylogeny in this study, we applied the partitioning scheme and partition-specific models identified by Castoe & Parkinson (2006). The Bayesian Markov chain Monte Carlo (BMCMC) estimate of the phylogeny was inferred using MrBayes ver. 3.1 (Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003) with default priors. As per the defaults, two parallel BMCMC runs were executed simultaneously and each was run for 5×10^6 generations. Parameters among partitions were unlinked, as was the rate of evolution (using the `ratepr = variable` command). Based on diagnostics described by Castoe & Parkinson (2006), both runs appeared stationary prior to 10^6 generations, and we conservatively excluded the first 1.5×10^6 generations of each run as burn-in. All post-burn-in estimates (sampled every 1000 generations) were combined, and phylogeny and parameter estimates were summarized from this combined posterior distribution. We also tested the alternative phylogenetic placement of *Bothriechis lateralis* as the sister lineage to *Bothriechis bicolor* (Crother *et al.*, 1992) using the Shimodaira–Hasegawa (SH) test (Shimodaira & Hasegawa, 1999) and the approximately unbiased (AU) test (Shimodaira, 2002) implemented in the program CONSEL (Shimodaira & Hasegawa, 2001).

Divergence time estimation

We used two relaxed clock methods to estimate divergence times across the pitviper phylogeny, the penalized likelihood (PL) method implemented in *r8s* (Sanderson, 1997, 2002, 2003) and the Bayesian relaxed clock method implemented in the program MULTIDISTRIBUTE (Thorne *et al.*, 1998; Thorne & Kishino, 2002). For the PL estimate, we estimated divergence times using *r8s* and then obtained confidence intervals on these dates using bootstrapped versions of the data set. To estimate PL confidence intervals, 1000 bootstrap replicates were generated using the program *BOOTSEQ* (Felsenstein, 2005). Branch lengths for each replicate data set were estimated using the GTR+ Γ +I model in *PAUP* ver. 4.10b (Swofford, 2001). Trees (and branch lengths) from the bootstrapped data sets were run in *r8s* and confidence intervals were summarized from this distribution using the Perl scripts provided at http://www.bergianska.se/index_kontaktaoss_torsten.html.

For the Bayesian inference of divergence times in MULTIDISTRIBUTE, we partitioned the molecular data by gene (four partitions) for all analyses. Using the program *baseml* (PAML package; Yang, 1997), model parameters were estimated using the model F84+ Γ for each partition. From this, branch lengths and the variance–covariance matrix were calculated using the program *estbranches*. Estimates of evolutionary rates and divergence times were then estimated using the program *multidivtime*. The priors used for analyses in *multidivtime* included: `rttm = 1.6`, `rtmsd = 0.2`, `rtrate = 0.3`, `rtrateds = 0.3`, `brownmean = 0.5`, `brownsd = 0.5` and `bigtime = 3.0`. The remaining priors used in *multidivtime* analyses were set to the program's default. Because the performance of divergence time-estimation approaches used here rely heavily on accurate branch-length estimation, divergence estimates are extremely

sensitive to short internodes that may have estimation variance that includes negative values of length or time. To avoid this potential problem, only subsets of the entire phylogenetic data set were used for r8s and MULTIDISTRIBUTE analyses. For both analyses, the topology was pruned to include only phylogenetically distinct lineages, thereby excluding lineages or samples that were associated with extremely small (near zero) branch lengths (as per the suggestions of both programs).

Calibration points

Because branch lengths represent the product of evolutionary rate and time, calibration points are necessary to separate these two underlying parameters and obtain an estimate of divergence times (Thorne & Kishino, 2005). We used four calibration points as minimum constraints to obtain date estimates for the pitviper phylogeny. In both the PL and Bayesian divergence analyses, we constrained the minimum ages of two temperate North American lineages based on fossil data: the origin of *Sistrurus* at 9 Ma (Parmley & Holman, 2007) and the origin of *Agkistrodon piscivorus* at 4.7 Ma (Holman, 2000). Because the PL method requires the age of one node to be fixed, for PL we fixed the age of the divergence between the two North American rattlesnake species *Crotalus ruber* and *Crotalus atrox* at 3.2 Ma (Castoe *et al.*, 2007). The divergence between these two species is thought to have occurred due to a well dated Pliocene marine incursion of the

Sea of Cortez, and is generally well corroborated across other taxa (for discussion see Castoe *et al.*, 2007). In the PL analyses, we also constrained the split between New World and Old World pitvipers as a minimum age at 16 Ma based on two sources of evidence: the oldest fossil of a viper found in the New World (Holman, 1977, 2000), and the end of the thermal optimum in the Miocene (Böhme, 2003; see also Burbrink & Lawson, 2007). For the Bayesian estimates of dates, the split between Old and New World pitvipers was used as the prior rttm; based on the evidence mentioned above, the rttm prior was set to 16 Myr and the standard deviation for that prior (rttmsd) to ± 4 Myr. The *C. atrox/ruber* split was also added as a constraint to the Bayesian estimation, set as 2.9–3.5 Myr.

RESULTS

Phylogenetic estimate

Our estimate of pitviper phylogeny is extremely similar to those of recent studies (Wüster *et al.*, 2002; Castoe *et al.*, 2005; Castoe & Parkinson, 2006), which was expected because the majority of the data and analytical approaches are the same. To maintain focus on groups of interest, we show summarized relationships among New World genera (Fig. 2) as well as detailed results only for genera of interest (Figs 2–4). We found strong support for the monophyly of all New World pitvipers (posterior probability, PP = 100), as well as a clade

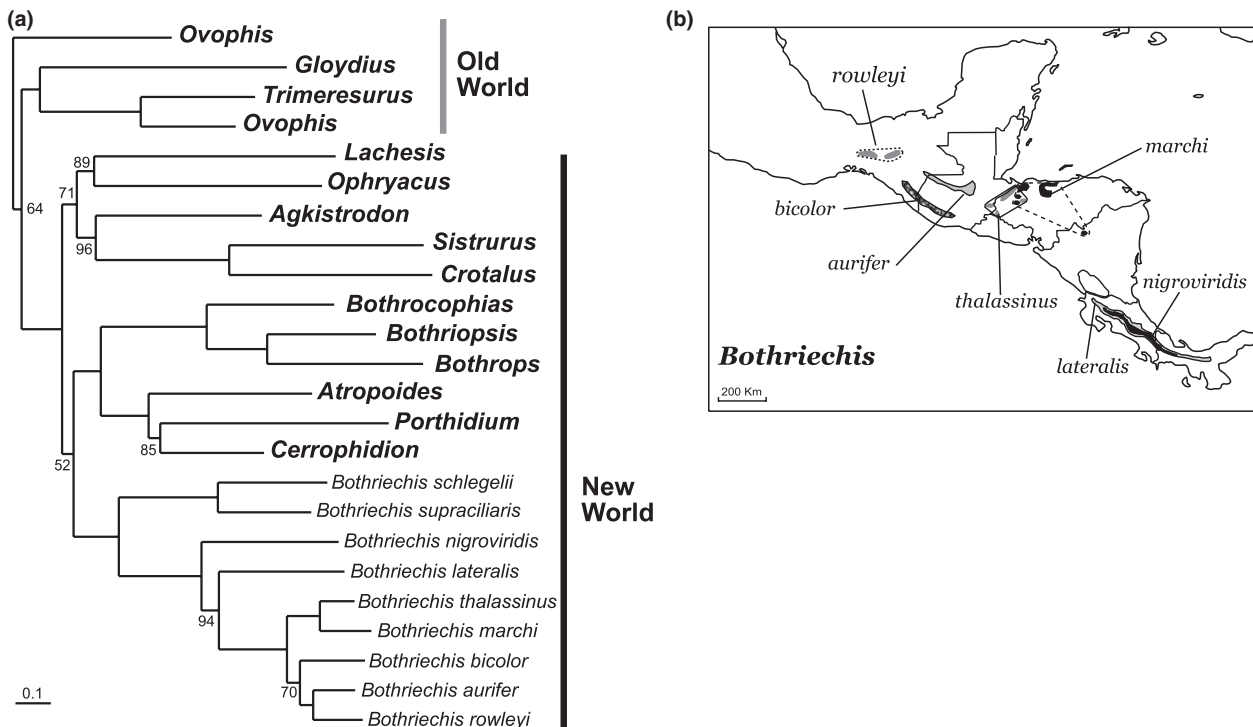


Figure 2 (a) Summary of Bayesian phylogenetic estimates of relationships among New World pitviper genera and relationships among species of the genus *Bothriechis*. All nodes shown received Bayesian posterior probabilities of 100% unless annotated on the tree. (b) Geographical distribution of Middle American highland species of the genus *Bothriechis* based on Campbell & Lamar (2004).

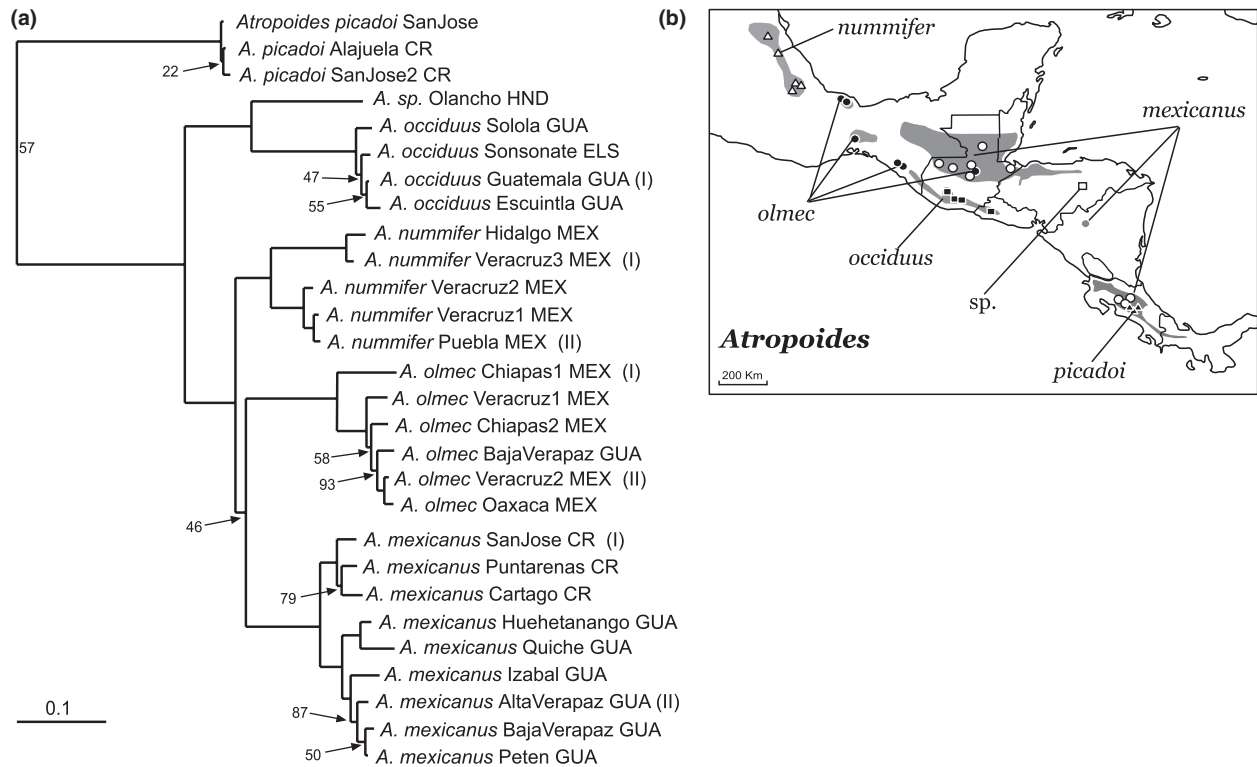


Figure 3 (a) Bayesian phylogenetic estimate of relationships among members of the genus *Atropoides*. All nodes shown received Bayesian posterior probabilities of 100% unless annotated on the tree. Roman numerals to the right of taxon names indicate individuals used for divergence dating, and correspond to those in Fig. 5. (b) Geographical distribution of *Atropoides* species. Shaded areas represent the known distribution for each species based on Campbell & Lamar (2004); dots correspond to the geographical origin of samples used for the molecular analyses.

representing the temperate genera *Crotalus*, *Sistrurus* and *Agkistrodon* (PP = 96; Fig. 2). Intergeneric relationships among Neotropical lineages match those determined by Castoe & Parkinson (2006), and include a large South American group (*Bothrops*, *Bothriopsis* and *Bothrocophias*) strongly supported as the sister clade of the Middle American *Porthidium* group (*Atropoides*, *Cerrophidion* and *Porthidium*). As in previous studies, *Bothriechis* was inferred to be the sister group to this Middle and South American assemblage (Fig. 2).

Monophyly of each genus of interest was inferred, with strong support for *Cerrophidion* and *Bothriechis* (PP = 100), and weaker support for *Atropoides* (PP = 57; Fig. 3). Relationships among all nominal species and major lineages within each of these genera were well resolved, with strong support in most cases. The new sequences of *Atropoides* and *Cerrophidion* added in this study illuminate substantial genetic structure within species. In *Atropoides* (Fig. 3), all species except *A. picadoi* and *Atropoides* sp. appear to contain substantial genetic diversity below the species level. We found substantial genetic structure within *Cerrophidion godmani*, consisting of at least four distinct and divergent clades (C1–C4; Fig. 4) that correspond to four main geographical components of the range of this species (Fig. 4b). Like Castoe & Parkinson (2006), we found strong support (Fig. 2a) for *B. lateralis* forming the sister group to the northern Central American highland

Bothriechis species, counter to the estimate that *B. lateralis* is the sister lineage to *B. bicolor* (Crother et al., 1992; see also Taggart et al., 2001). The sister-lineage relationship between *B. lateralis* and *B. bicolor* was also rejected by SH tests ($P < 0.001$) and AU tests ($P < 0.001$). These results provide strong evidence in support of the topology with *B. lateralis* as the sister group to all northern Central American highland *Bothriechis* species (as in Fig. 2a).

Divergence times

Estimates of divergence times were generally similar between the two divergence dating methods used (Table 1). The most notable contrast between the two sets of estimates was a substantial difference in confidence intervals, with the PL intervals being narrower and symmetrically distributed around the mean, whereas the Bayesian estimates had broader confidence intervals that were asymmetrical and skewed towards more ancient divergence times. This contrast between Bayesian and PL estimates has been noted elsewhere, and some have suggested that the current method of obtaining bootstrap-based intervals in PL can produce confidence interval distributions that are improperly uniform and overly narrow (Burbrink & Pyron, 2008). Thus, the credible intervals of Bayesian estimates are thought to be more accurate in their

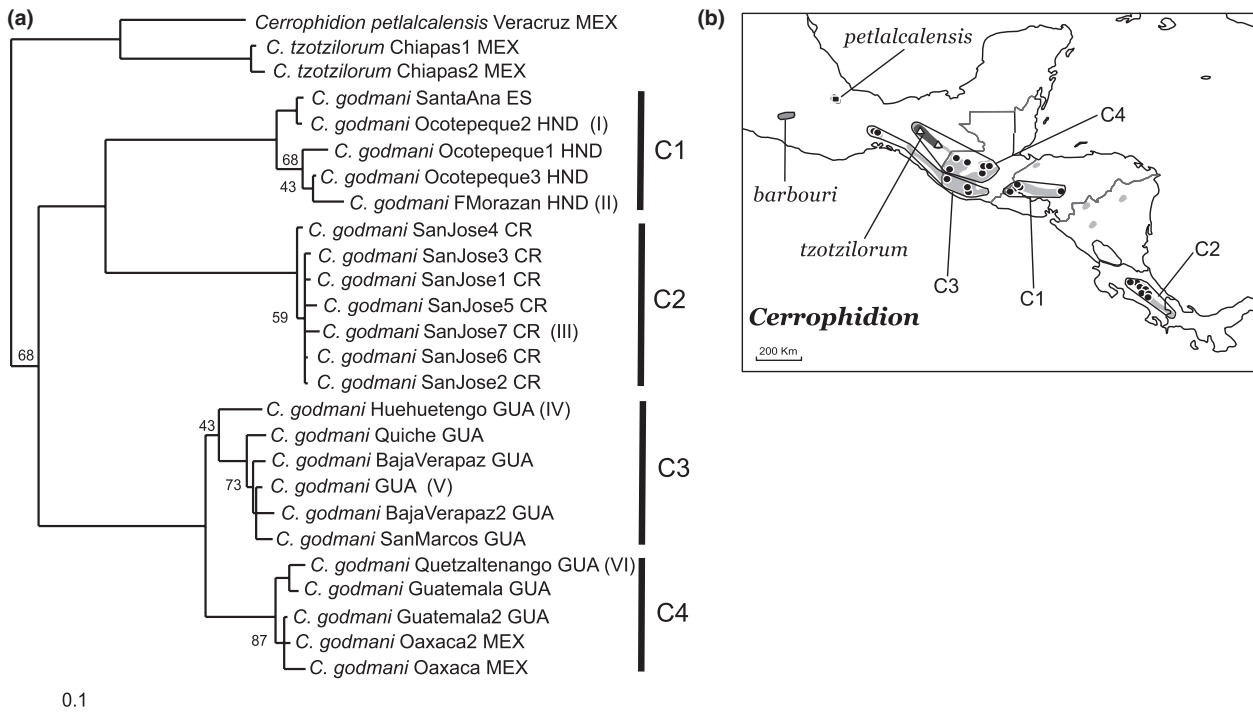


Figure 4 (a) Bayesian phylogenetic estimate of relationships among members of the genus *Cerrophidion* included in this study. All nodes shown received Bayesian posterior probabilities of 100% unless annotated on the tree. Roman numerals to the right of taxon names indicate individuals used for divergence dating, and correspond to those in Fig. 5. (b) Geographical distribution of *Cerrophidion* species in Middle America. Shaded areas represent the distribution for each species based on Campbell & Lamar (2004); dots correspond to the geographical origin of samples used for the molecular analyses. Major phylogeographical lineages within *Cerrophidion godmani* are labelled C1–C4 (a,b), and are indicated by polygons on the map (b).

Table 1 Estimates of divergence times (Ma) for major events in New World pitviper lineages.

Node	BI			PL		
	Mean	Lower	Upper	Mean	Lower	Upper
Origin of New World pitvipers	16.08	14.33	17.99	17.35	16.15	18.55
Origin of Bothropoid group	12.82	10.67	15.15	14.15	13.13	15.17
Origin of <i>Atropoides</i>	9.95	8.13	12.02	10.76	9.98	11.54
Origin of <i>Bothriechis</i>	14.1	11.99	16.29	15.24	14.25	16.23
Origin of <i>Cerrophidion</i>	9.43	7.66	11.47	10.41	9.65	11.17
(1) Nicaragua						
<i>Atropoides</i>	8.56	6.77	10.61	9.28	8.47	10.09
<i>Bothriechis</i>	7.67	5.73	9.87	8.04	7.35	8.74
<i>Cerrophidion</i>	4.39	3.06	6.03	4.03	3.54	4.53
(2) Motagua–Polo chic						
<i>Atropoides</i>	4.82	3.55	6.35	4.69	4.25	5.13
<i>Bothriechis</i>	4.56	3.3	6.03	4.5	4.08	4.92
<i>Cerrophidion</i>	5.73	4.31	7.37	5.51	4.97	6.04
(3) Tehuantepec						
<i>Atropoides</i>	3.05	2.18	4.15	3.29	2.96	3.63
<i>Bothriechis</i>	3.49	2.44	4.72	3.05	2.68	3.42
<i>Cerrophidion</i>	3.31	2.16	4.67	2.94	2.54	3.35

Mean estimates of divergence times based on Bayesian inference (BI) and penalized likelihood (PL) are given with corresponding upper and lower bounds of the 95% credibility (BI) or confidence intervals (PL) for each estimate.

breadth and skew in contrast to PL bootstrap-based intervals. Because of this potential bias in the PL estimates, we report results based primarily on the Bayesian estimates and 95%

credible intervals, and comment on the PL estimates where relevant. Direct comparisons of the results of both methods are given in Table 1.

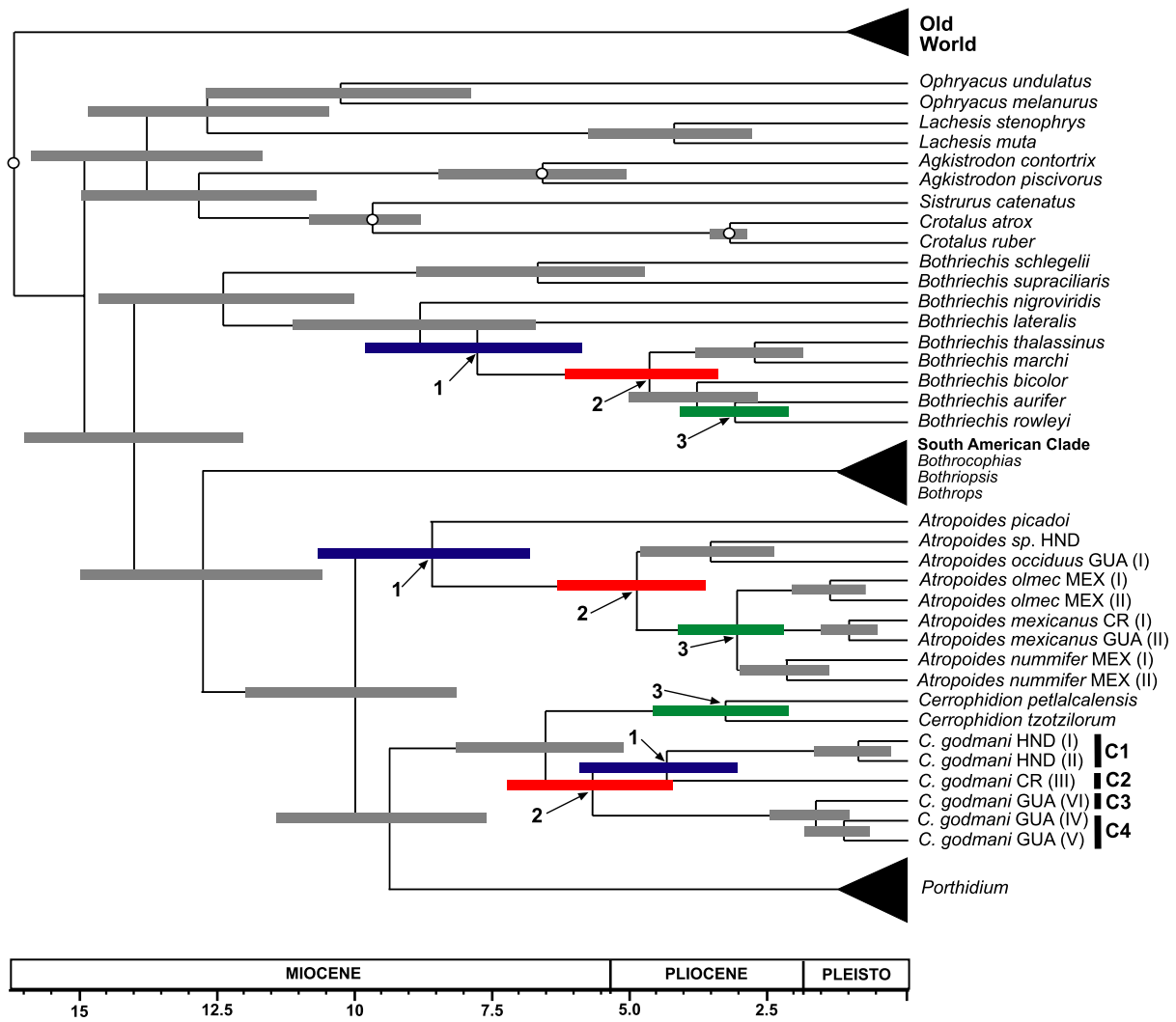


Figure 5 Bayesian estimates of divergence times (Ma) for the pitviper phylogeny. The mean estimate is represented by the node; bars represent 95% credibility intervals for divergence estimates; open circles represent calibration points described in the text. Numbers on nodes (1–3) correspond to the biogeographical breaks for highland taxa: (1) Nicaragua Depression; (2) Motagua–Polochic Fault valleys; (3) Isthmus of Tehuantepec. Roman numerals are used to cross-reference (with Figs 3 & 4) individuals per species used in divergence estimation.

All intergeneric divergences within the New World pitvipers are estimated to have occurred during the Miocene, and the New World lineage is estimated to have diverged from Old World pitvipers in the early Miocene, between 14 and 18 Ma (Table 1; Fig. 5). The majority of cladogenetic events that gave rise to the current genera and most of the species occurred in the middle–late Miocene and early Pliocene. The three genera we focus on here are inferred to have arisen from the middle to late Miocene (Table 1; Fig. 5). All nominal species of highland pitvipers appear to have diverged prior to the late Pliocene, predominantly from late Miocene to middle Pliocene (Fig. 5). Major divergences within highland pitviper species occurred over a broad period of time (early Pliocene–Pleistocene; Fig. 5). Phylogroups within the wide-ranging species *C. godmani* began to diverge in the late Miocene (*c.* 5.7 Ma) and continued to do so through the

Pliocene and Pleistocene, before the divergence of many other lineages of Neotropical pitviper species from their sister groups (Fig. 5). Intraspecific phylogroups within *Atropoides* diverged at the end of the Pliocene and the Pleistocene (2.1–0.9 Ma; Fig. 5).

Three major phylogeographical divergence events that have occurred in each of the three genera of interest show different levels of temporal correspondence; these are labelled as 1–3 in Fig. 6. For the first phylogeographical break at the Nicaraguan Depression (labelled split 1; Fig. 6), *Bothriechis* and *Atropoides* lineages show strong overlapping temporal divergence (Table 1; Fig. 6) in the middle–late Miocene, whereas the corresponding geographical split in *Cerrophidion* is substantially later, in the early–middle Pliocene (Figs 5 & 6; Table 1). The posterior probability distributions of divergence times in the first two genera broadly overlap, but show almost no

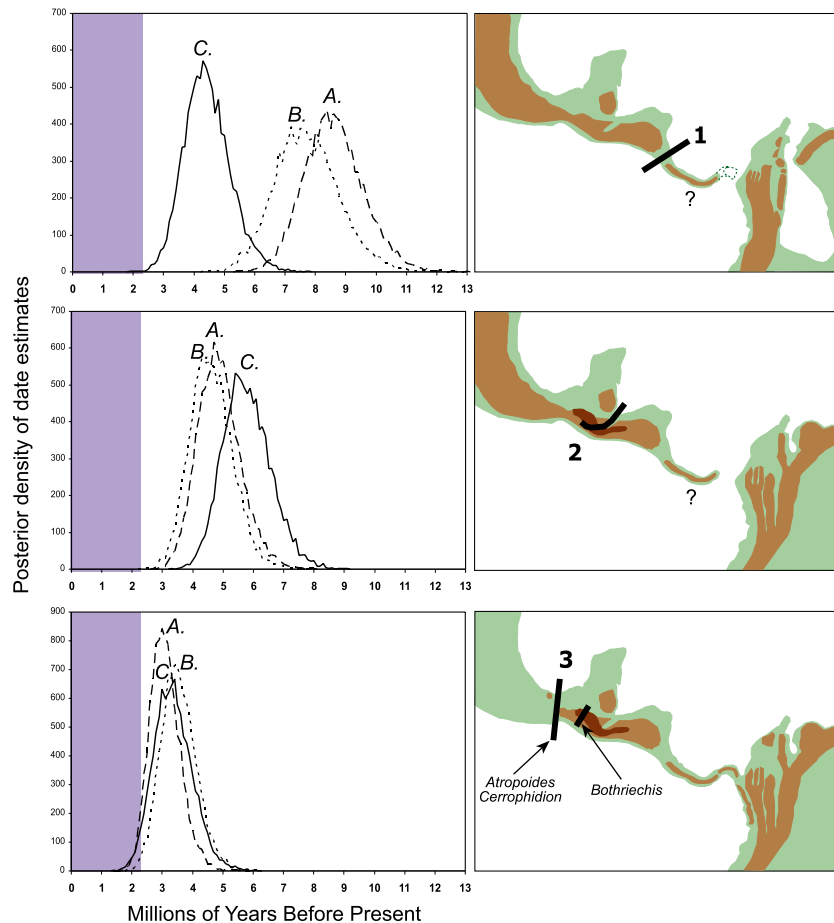


Figure 6 Bayesian posterior densities for divergence time estimates of the three highland genera across three major biogeographical breaks. Letters over the distributions indicate the three genera (A. = *Atropoides*, B. = *Bothriechis*, C. = *Cerrophidion*). Shaded regions indicate period of glacial cycles in the late Pliocene through the Pleistocene. Figures on the right show the biogeographical break and the potential approximate palaeogeographical reconstruction at that time; darker shading indicates major highland masses in palaeogeographical reconstructions. Palaeogeographical reconstruction in Lower Central America based on Kirby & MacFadden (2005; see discussion for other reconstructions).

overlap with that of *Cerrophidion* (Fig. 6), suggesting that *Atropoides* and *Bothriechis* had undergone an essentially coordinated divergence that was not shared with *Cerrophidion*. For the second major divergence event, across the Motagua–Polochoic Fault, there is strong evidence for the shared divergence between *Atropoides* and *Bothriechis*, also with moderate evidence for this divergence being shared by *Cerrophidion* (Table 1; Figs 5 & 6). Posterior probability distributions of divergence times for all three genera do largely overlap across the period of c. 4–5.5 Ma, providing evidence that they experienced a mostly simultaneous divergence at the Motagua–Polochoic Fault in the late Miocene–early Pliocene (Fig. 6).

The third major phylogeographical break, across the Isthmus of Tehuantepec, provides particularly strong evidence of a shared simultaneous divergence across the three genera in the middle Pliocene (Table 1; Figs 5 & 6). The posterior probability distributions of divergence time estimates are nearly identical between *Atropoides* and *Cerrophidion*, which show a divergence at the geographically defined Isthmus of Tehuantepec. Although *Bothriechis* does not occur north of the geographical Isthmus, the divergence of *Bothriechis rowleyi* (from *Bothriechis aurifer*) directly adjacent to the Isthmus shows nearly perfect temporal correspondence with the breaks in the other two genera (Fig. 6). Below we elaborate on geological evidence suggesting that the break observed in

Bothriechis adjacent to the Isthmus of Tehuantepec may be geologically tied to the events leading to divergence in the other two genera in this region.

DISCUSSION

A consensus of ancient Middle American highland speciation

Glacial climatic cycles during the late Pliocene–Pleistocene, subsequent to the establishment of the late Pliocene land connections between Middle and South America, have been viewed as the predominant processes that have generated substantial Middle American biodiversity, particularly for highland taxa (Savage, 2002, and references therein). In general, this also has been the dominant hypothesis for explaining highland pitviper speciation – both Crother *et al.* (1992) and Castoe *et al.* (2003) focused on the period from the middle Pliocene and later, and on climatic fluctuations, as having driven speciation in *Bothriechis* and *Atropoides*, respectively. Despite consensus in the identification of major biogeographical boundaries that have shaped the region's biodiversity (Savage, 1982; Morrone, 2001), there has been little quantitative insight as to when these barriers may have led to diversification, and in what temporal order. This study contributes three new important findings that reject previous

hypotheses and clarify historical biogeographical patterns in Middle American highland taxa.

First, estimates of pitviper phylogeny and divergence times for Middle American highland lineages reject Savage's model of highland speciation in which late Pliocene and Pleistocene climatic changes are major determinants of current species diversity (Savage, 2002). Instead, our results suggest that Miocene–Pliocene tectonic activity played a dominant role in generating regional highland species biodiversity. This conclusion contrasts with the majority of previous suggestions by taxon-specific studies on pitvipers (Crother *et al.*, 1992; Castoe *et al.*, 2003; Werman, 2005); plants alone (Burnham & Graham, 1999); and plants, insects and fish (Marshall & Liebherr, 2000). This and other recent studies highlight the significance of pre-Pliocene diversification in Middle America (Smith *et al.*, 2007; Wiens *et al.*, 2007), together with ancient faunal interchange between Middle and South America (Bermingham & Martin, 1998; Barraclough & Vogler, 2002; Wüster *et al.*, 2002; Parra-Olea *et al.*, 2004; Pennington & Dick, 2004; Steppan *et al.*, 2004; Crawford & Smith, 2005; Concheiro-Pérez *et al.*, 2007; Koepfli *et al.*, 2007; Wahlberg & Freitas, 2007).

Second, there is evidence for a congruent temporal pattern of divergence across three different lineages of Middle American highland pitvipers, corresponding to major geographical breaks among Middle American highland masses. This, to our knowledge, is the first evidence of a clear pattern of temporal and spatial congruence in divergence patterns across multiple highland lineages of any taxon in Middle America. This example therefore provides one of the first explicit predictive models for speciation in this heavily studied epicentre of biodiversity. These biogeographical break points are obvious contemporary barriers for highland species and have been the focus of previous biogeographical attention (Savage, 1982, 1987; Campbell, 1999; Duellman, 1999; Sullivan *et al.*, 2000; Morrone, 2001), but no clear evidence or consensus for when and how these regions have broadly shaped biodiversity has previously emerged. It is also significant to bear in mind an important strength of our analyses – regardless of the exact estimates of absolute divergence times, our inference of relative temporal congruence among lineage divergences is particularly robust because all estimates are derived from a single, large, dated tree (Thorne & Kishino, 2005). Thus, the evidence in this study regarding the relative correspondence of divergence times across multiple lineages of pitvipers is robust and fairly independent of the accuracy of the absolute estimates of divergence times.

Third, we do find evidence that climatic changes associated with the onset of glacial cycles in the late Pliocene–Pleistocene may have led to lineage diversification in Middle American highland pitvipers, but only among populations within species. This evidence is consistent with glacial climatic cycles contributing to the fragmentation of once contiguous highland habitats, leading to the subsequent divergence among populations of *Atropoides* and *Cerrophidion*. These inferences provide new insight into corridors of highland habitat that

at one time facilitated gene flow that may have been fragmented due to climatic changes in the late Pliocene and Pleistocene.

Below, we first discuss evidence from this study for three shared ancient (Miocene–Pliocene) divergences across Middle American highland pitviper lineages, and the underlying tectonic and biogeographical hypotheses surrounding these divergences. Next we focus on the intraspecific sampling of *Atropoides* and *Cerrophidion* and the evidence for late Pliocene–Pleistocene effects on lineage diversification, and we examine previous biogeographical hypotheses for *Bothriechis* species.

Shared divergence (1): the Nicaraguan Depression

The lowland area known as the Nicaraguan Depression is the geological result of a back-arc formation that has continued to evolve for the past 10 Myr (Rogers *et al.*, 2002; Marshall, 2007). This region separates two highland masses, the Chortis block highlands (Honduras and Nicaragua) to the north, and the Lower Central American highlands of Costa Rica and Panama. Evidence suggests that a marine gap existed between the Chortis and Lower Central American highlands during the Miocene and the majority of the Pliocene (Coates & Obando, 1996; Iturralde-Vinent & MacPhee, 1999; Iturralde-Vinent, 2006). Alternatively, Kirby & MacFadden (2005) have suggested that a narrow landmass connected modern-day Honduras and Costa Rica during this time. The Nicaraguan Depression has been identified as a major phylogeographical break for many taxa, including frogs (Savage, 1987; Duellman, 1999), salamanders (Parra-Olea *et al.*, 2004), snakes (Savage, 1982; Cadle, 1985), birds (Pérez-Emán, 2005), and plants, insects and fish (Marshall & Liebherr, 2000; Halas *et al.*, 2005).

Middle American highland pitvipers also provide strong support for this region representing a major historical barrier to gene flow. We found evidence for temporal congruence of highland pitviper divergence across this break in two of the three pitviper lineages. *Bothriechis* and *Atropoides* show broadly overlapping divergence estimates across this break in the middle–late Miocene, *c.* 7.7–8.6 Ma [Bayesian confidence intervals (BCIs) = 5.7–9.9 and 6.8–10.6 respectively, Fig. 6]. Although estimates of these two genera appear to indicate a fairly coincident divergence at the Nicaraguan Depression, the third genus, *Cerrophidion*, appears to have diverged across this region much later in the early–middle Pliocene, *c.* 4.4 Ma (BCI = 3.1–6.0 Ma, Fig. 6). The posterior probability distribution of *Cerrophidion* divergence times shows very little overlap with that of the other two genera (Fig. 6) and strongly suggests a unique biogeographical scenario for *Cerrophidion* divergence across this barrier.

The apparent lack of temporal correspondence of divergences between *Cerrophidion* and the other two genera may indicate that *Cerrophidion* has different dispersal capabilities, or that members of this genus may not have been distributed across the Depression in the middle Miocene. Of the three genera, *Cerrophidion* tends to inhabit the highest elevations

(up to *c.* 2700 m; Campbell & Lamar, 2004), and it has been suggested that high-elevation habitats may not have existed in lower Central America until the Pliocene (Coates & Obando, 1996). The estimate of more recent cladogenesis within *Cerrophidion* that is not observed in either *Atropoides* or *Bothriechis* is intriguing, and suggests that dispersal and vicariance of highland lineages across the Nicaraguan Depression has occurred multiple times in the Miocene–Pliocene. It is notable that these estimates of divergence times are collectively consistent with the model of Kirby & MacFadden (2005), corroborating their suggestion of a narrow landmass across the Nicaraguan Depression during the Miocene and Pliocene.

Shared divergence (2): the Motagua–Polochic Faults

The Motagua–Polochic Fault represents the contact zone between the Maya and Chortis tectonic blocks (Marshall, 2007). The eastward motion of the Chortis block that has continued since the Cretaceous is responsible for the generation of a majority of the mountain-building across south-western Mexico and Nuclear Central America (Rogers *et al.*, 2002). Numerous studies have suggested that this physiographical barrier caused phylogeographical breaks in different taxa (Humphries, 1982; Perdices *et al.*, 2002, 2005; Halas *et al.*, 2005; Devitt, 2006; Concheiro-Pérez *et al.*, 2007). For lowland-inhabiting snakes, Devitt (2006) estimated a cladogenetic event in this region at 7.7 Ma, and Perdices *et al.* (2005) found that freshwater eel-like synbranchid fishes diverged *c.* 11.2 Ma. In contrast, our estimates suggest divergence of highland lineages of pitvipers later in the Miocene and/or early Pliocene (Figs 5 & 6).

Our divergence time estimates show a geographically congruent, nearly simultaneous diversification scenario in the late Miocene, centred *c.* 4.1–5.0 Ma, for the three highland lineages of pitvipers (Figs 5 & 6). The correspondence between divergence times for *Atropoides* and *Bothriechis* is excellent (4.3 and 4.1 Ma, respectively), and it appears that *Cerrophidion* may have diverged slightly earlier (5.0 Ma; Figs 5 & 6). This result is consistent with the expectation that, because *Cerrophidion* is restricted to higher elevation habitats, gene flow may have been severed slightly earlier in this group compared with the other two lineages. It is interesting that there is fairly strong evidence for simultaneous divergence across highland lineages at this fault zone that contrasts substantially with more ancient divergence estimates for lowland groups (Perdices *et al.*, 2005; Devitt, 2006). The extensive mountain-building and physiographical reshaping of the region makes historical inferences difficult, but these results may indicate that this region has contributed to the divergence of lineages with different habitat requirements in markedly different ways over an extended period of time.

Shared divergence (3): the Isthmus of Tehuantepec

Geographically, the Isthmus of Tehuantepec is the narrow lowland region that separates the highlands of southern

Mexico (Sierra Madre Oriental and Sierra Madre del Sur) from the Chiapan-Guatemalan highlands of Nuclear Central America. This region is well known as a major biogeographical node where historical events have formed a transition between the Nearctic and the Neotropical biogeographical zones (Hallfater, 1987; Marshall & Liebherr, 2000; Morrone & Márquez, 2001). Biogeographical studies on specific taxa have found the Isthmus of Tehuantepec to be a phylogeographical barrier for highland species (Chippindale *et al.*, 1998; Sullivan *et al.*, 2000; León-Paniagua *et al.*, 2007). More recent studies on lowland species have revealed similar phylogeographical structure separating lineages on both sides of the Isthmus (Hasbún *et al.*, 2005; Devitt, 2006; Mulcahy *et al.*, 2006).

Tectonically, the Isthmus represents a visible marker for the three-way junction of tectonic plates that have remained extremely active in shaping the regional landscape since the Cretaceous. It is thought that a highland corridor spanning the Isthmus in the Miocene was subsequently destroyed due to extreme tectonic activity relating to the subduction of the Cocos Plate (Barrier *et al.*, 1998; Manea & Manea, 2006). Tectonic markers distributed both on the Isthmus of Tehuantepec and on surrounding upland areas show massive down-dropping of the Chiapan-Guatemalan region with respect to the areas to the north and west during the late Miocene–early Pliocene associated with faulting occurring across the short axis of the Isthmus, resulting in a significant reduction in elevation and subsequent marine inundations (Barrier *et al.*, 1998).

Atropoides and *Cerrophidion* each show clear phylogeographical breaks centred around the geographical Isthmus of Tehuantepec, and estimates of divergence times between these two genera show remarkable temporal congruence over this boundary. Our results suggest that these two genera experienced a simultaneous divergence across this zone in the Pliocene, *c.* 3.1–3.5 Ma (Fig. 6), consistent with geological evidence for a tremendous tectonic event in which highlands at the Isthmus were reduced to a submarine embayment over a short period in the Pliocene (Barrier *et al.*, 1998).

Unlike the other two genera, *Bothriechis* does not occur west of the Isthmus of Tehuantepec, although one species, *B. rowleyi*, is endemic to north-west Chiapas adjacent to the Isthmus (Fig. 2). *Bothriechis rowleyi* is distributed only in the mountain region of northern Chiapas, a recent geological formation called the Modern Chiapas Volcanic Front (Manea & Manea, 2006). Around 3 Ma, the continued slab subduction of the Cocos plate generated extensive orogenic changes not only at the Isthmus proper, but also in surrounding regions that led to the uplift of the Modern Chiapas Volcanic Front (Manea & Manea, 2006). It is therefore reasonable to infer that the final formation of the Chiapas highlands during the Pliocene, associated with tectonic activity at the triple plate junction at the Isthmus, led to the vicariance between the ancestors of *B. rowleyi* and its sister species *B. aurifer* (Fig. 6). The temporal congruence between this divergence in *Bothriechis* and that of the other two genera at almost exactly 3 Ma is impressive, and suggests that these vicariant events were nearly simultaneous and possibly driven by the same tectonic activity surrounding

the Isthmus. Although strongly supported by geological data, this is the first evidence of which we are aware that demonstrates a potential temporal (and tectonic) link between evolutionary vicariance events at the Isthmus of Tehuantepec and those in the neighbouring Chiapan highlands. Future research to increase the resolution of biogeographical analysis in the Isthmus region may provide tests of this hypothesis, while further illuminating the complex role of this biogeographical node in shaping historical gene flow between the Nearctic and Neotropical regions.

Intraspecific phylogeography of *Atropoides* and *Cerrophidion*

New fine-scale sampling of *Atropoides* and *Cerrophidion* highlights substantial genetic structuring (Figs 3 & 4) which was estimated to have occurred during the Pliocene and Pleistocene. Within *Atropoides* species, Pleistocene divergences are estimated (1) within the Sierra Madre Occidental in eastern-central Mexico (*Atropoides nummifer*), (2) across the Nicaraguan Depression (*Atropoides mexicanus*), and (3) across the Isthmus of Tehuantepec (*Atropoides olmec*). Similarly to *Atropoides*, there is evidence that some among-population gene flow in *C. godmani* may have been affected by glacial climatic cycles in the Pleistocene. The divergence of phylogroups C3 and C4 (Fig. 4), representing the separation of the Northeastern from the Southwestern Guatemalan highlands, appears to have occurred at the temporal boundary between the Pliocene and Pleistocene (Fig. 5). Further divergences across highlands in eastern Honduras (within C1), and among interior Guatemalan highlands (within C4) may also have been associated with Pleistocene climatic change (Fig. 5).

New sampling within *Atropoides* and *Cerrophidion* also provides insight into previous biogeographical and taxonomic hypotheses. Castoe *et al.* (2003) hypothesized that a recent corridor for gene flow extended across the Isthmus of Tehuantepec to explain the close relationship between populations of *A. olmec* in Veracruz, Mexico and Baja Verapaz, Guatemala; new *Atropoides* samples from Chiapas, Mexico, that are associated with *A. olmec* further support this. Our new sampling of *C. godmani* demonstrates an extensive amount of ancient genetic structure, which has generally been suggested previously (Castoe *et al.*, 2003, 2005). Estimates of divergence times also suggest that the species *C. godmani* began to diversify prior to some major clades of *Atropoides* (all except *A. picadoi*) and *Bothriechis* (all northern highland species; Fig. 5). The question of whether major phylogeographical clades of *C. godmani* may warrant recognition as distinct species is currently being evaluated as part of our ongoing studies of the biodiversity and historical biogeography of this region.

Alternative hypotheses for biogeography of *Bothriechis*

Modern biogeographical hypotheses rely on the synthesis of multiple layers of inference, including distribution patterns of

lineages, phylogeny and divergence time estimates, rendering biogeographical inferences sensitive to all these underlying estimates. The phylogeny of *Bothriechis* has been controversial (Crother *et al.*, 1992; Taggart *et al.*, 2001; Castoe & Parkinson, 2006) largely because a previous study (Taggart *et al.*, 2001) suggested that conflicting phylogenetic estimates from morphology plus allozymes vs. mitochondrial gene sequences indicated that mitochondrial introgression and/or incomplete lineage sorting may confound mitochondrial gene phylogenies of the group. Based on allozyme and morphological data, Crother *et al.* (1992) suggested that *B. lateralis* was phylogenetically nested within northern Middle American highland lineages (sister to *B. aurifer*), rather than the sister group to all northern highland species, as in our phylogenetic placement (Fig. 2). Based on our mitochondrial DNA data set, the former hypothesis was strongly rejected by SH and AU tests ($P \ll 0.001$) in favour of the relationships recovered in our tree (Fig. 2). Our mitochondrial phylogeny of *Bothriechis* and that of the combined data of Taggart *et al.* (2001: Fig. 6b) are essentially the same; both place *B. lateralis* as the sister lineage to the northern highland species. The conclusion of Taggart *et al.* (2001), however, was that the mitochondrial tree was incorrect because it differed from a tree estimated from morphological and allozyme characters (from Crother *et al.*, 1992). In unpublished analyses, we have examined multiple nuclear genes and sampled mitochondrial haplotypes extensively within species of *Bothriechis*, and all available data have failed to reveal evidence of incomplete lineage sorting, hybridization or mitochondrial vs. nuclear gene tree incongruence (C.L. Parkinson, T.A. Castoe and J.M. Daza, unpublished data). These data suggest that the nuclear gene tree (and presumed organismal phylogeny) is consistent with our mitochondrial phylogeny estimate.

Based essentially on geological evidence, Crother *et al.* (1992) suggested that the diversification of Middle American highland *Bothriechis* species spanned a broad time period, from the Eocene–Oligocene boundary (middle Tertiary) through the Pleistocene. In contrast, our estimates from relaxed molecular clock models suggest that this group diversified over a more contracted period, ranging from the middle Miocene through late Pliocene (Fig. 5). Despite this contrast, both hypotheses share most inferences of geographical boundaries and related geological and tectonic events that historically underlie phylogenetic splits within *Bothriechis*. Because their phylogeny estimate places *B. lateralis* within the northern highland species group, however, Crother *et al.* (1992) suggested recent southward dispersal of the ancestor of *B. lateralis* from northern Middle America to Costa Rica. In contrast, our biogeographical model essentially depicts a more simplistic northward progression of cladogenesis that requires no inference of dispersal and is more compatible with the patterns we observed in *Atropoides* and *Cerrophidion*, suggesting vicariance as the primary driving force underlying speciation. Unlike the other two genera in this study, *Bothriechis* appears to have diversified (into *B. nigroviridis* and *B. lateralis*) early within Lower Central America during the middle–late Miocene. This

divergence also is associated with a shift in altitudinal habitat, as *B. lateralis* typically occupies lower elevations than does *B. nigroviridis* (Campbell & Lamar, 2004). Despite this uniqueness, our revised model for *Bothriechis* biogeography yields exceptional temporal and phylogeographical correspondence between patterns in this group with those estimated for *Atropoides* and *Cerrophidion*.

CONCLUSIONS

The species-level biodiversity of Middle American highland pitvipers, as currently recognized, appears to have been generated predominantly by tectonic events occurring during the Miocene and Pliocene, independent of Pleistocene climatic fluctuations. We do, however, find evidence that the onset of glacial cycles may have had an impact on highland pitviper lineage diversity, but only within species. Although future taxonomic revisions (e.g. in *C. godmani*) may alter this general conclusion, evidence that the current high taxonomic diversity of pitvipers in the region owes its origins to events that predate the Pleistocene is both significant and impressive. We have identified several major historical events, each of which appears to have resulted in the simultaneous vicariance and diversification of multiple highland lineages in Middle America. These findings suggest that Miocene and Pliocene events may have broad predictive power across entire communities of highland-distributed organisms. Inferences from highland pitviper lineages show a strong underlying pattern of south to north, Miocene–Pliocene vicariance across highland masses that can be explicitly examined as a baseline hypothesis for other taxa. This new evidence suggests the existence of an underlying and unifying model of Middle American biogeography. It is therefore a strong motivation for future comparative phylogeographical work in the region, and suggests that a cohesive hypothesis of the region's history may eventually be unveiled through the comparative phylogeography of its biodiversity. The complex and controversial geological and tectonic history of Middle America has posed a substantial challenge for palaeogeography and biogeography. Further comparative biogeographical research may thus present tremendous potential for both generating and testing hypotheses leading to the formulation of a synthetic physical and biotic inference of the region's history and evolution.

ACKNOWLEDGEMENTS

We thank the many people who over the years have contributed insight and suggestions that have added to this study, including: B. Crother, T. Doan, J. Meik, B. Noonan, J. Reece, W. Schargel and C. Spencer. Andrew Crawford is also thanked for constructive advice and conversation, including discussion of his unpublished work. Manuel Iturralde-Vinent provided critical literature and insights regarding Caribbean palaeogeography. Several researchers provided tissues under their care and obtained during sponsored research, including Laurie Vitt (University of Oklahoma, obtained through NSF

grants DEB-9200779 and DEB-9505518), Cesar Jaramillo (Círculo Herpetológico de Panamá), William Duellman, Linda Trueb and Eli Greenbaum (University of Kansas), Robert Murphy (Royal Ontario Museum), Marcio Martins (Universidade de São Paulo) and Gunther Köhler (Forschungsinstitut Senckenberg). This research was supported by a UCF startup package to C.L.P., a grant from Bioclon to E.N.S., an NSF Collaborative Research grant to C.L.P., E.N.S. and J.A.C. (DEB-0416000, 0416160), as well as NSF grants DEB-0613802 and DEB-9705277 to J.A.C. T.A.C. was also supported by an NIH training grant (LM009451) while finalizing this project, and U.K. by the LOEWE programme of the state government of Hessen.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Appendix S1 Sequences used in phylogenetic and divergence time estimation, with Genbank numbers and voucher information. Sequences added specifically in this study are indicated in bold.

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BIOSKETCHES

Todd A. Castoe and **Juan M. Daza** performed this research collaboratively while they were doctoral students in the laboratory of Christopher L. Parkinson at the University of Central Florida, building on their shared interests in snake genomics, molecular evolution, phylogenetics, molecular dating and Neotropical biogeography. Todd Castoe is now a postdoctoral fellow and Associate Director of the Consortium for Comparative Genomics at the University of Colorado School of Medicine.

Editor: David Hafner